

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(S1) International Patent Classification 6 : C07K 14/47, A23L 1/305, A61K 38/17	A1	(11) International Publication Number: WO 97/24371 (43) International Publication Date: 10 July 1997 (10.07.97)		
(21) International Application Number: PCT/EP96/05846		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, LZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).		
(22) International Filing Date: 27 December 1996 (27.12.96)				
(30) Priority Data: RM95A000850 27 December 1995 (27.12.95) IT				
(71) Applicant (<i>for all designated States except US</i>): MIDIA LIMITED [--]; 1st floor, Channel House, Green Street, St. Helier, Jersey JE4 5UW (GB).	Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>			
(72) Inventor; and				
(75) Inventor/Applicant (<i>for US only</i>): POZZILLI, Paolo [IT/IT]; Via Vallombrosa, 40, I-00135 Rome (IT).				
(74) Agent: GERVASI, Gemma; Notarbartolo & Gervasi, Corso di Porta Vittoria, 9, I-20122 Milan (IT).				
(54) Title: PRODUCT DERIVED FROM MILK SUBSTANTIALLY FREE OF BETA CASEIN FROM NON-HUMAN MAMMALS AND RELATIVE USE				
(57) Abstract				
The present invention is related to a product derived from milk, substantially free of beta casein from non-human mammals. The invention is also related to the use of such a product especially in relation to diet, more particularly for early infancy, in the prevention of insulin-dependent diabetes.				

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IR	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FJ	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/EP 96/05846

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/47 A23L1/305 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A23L A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 17518 A (INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE) 29 June 1995 see figure 20 ---	1
X	WO 94 06306 A (NEW ZEALAND DAIRY RESEARCH INSTITUTE) 31 March 1994 cited in the application see the whole document ---	1-17
X	WO 91 02539 A (INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE) 7 March 1991 cited in the application see the whole document ---	1 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- 'A' document defining the general state of the art which is not considered to be of particular relevance
- 'E' earlier document but published on or after the international filing date
- 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

- 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- 'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- '&' document member of the same patent family

1

Date of the actual completion of the international search

Date of mailing of the international search report

13 May 1997

27.05.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+ 31-70) 340-3016

Authorized officer

Masturzo, P

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 96/05846

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 256, no. 8, 25 August 1984, MD US, pages 5132-5136, XP002030852 R GREENBERG ET AL.: "Human beta-casein" see figure 3 ---	1
X	COLLOQUE INSERM (2ND FORUM ON PEPTIDES), vol. 172, 1989, JOHN LIBBEY EUROTEXT LTD. , pages 65-68, XP002030853 J LÉONIL ET AL. : "Study of tryptic hydrolysis of bovine beta-casein with the aim of preparing peptides with biological activities in membrane reactors " see the whole document ---	1
X	CHEMICAL ABSTRACTS, vol. 118, no. 7, 15 February 1993 Columbus, Ohio, US; abstract no. 58380, XP002030855 see abstract & J DAIRY RES. , vol. 59 , no. 4, 1992, J LEAVER & A J R LAW: "Preparative-scale purification of bovine caseins on a cation-exchange resin " ---	13
P,X	WO 96 14577 A (NATIONAL CHILD HEALTH RESEARCH FOUNDATION/ NZ DAIRY BOARD) 17 May 1996 see the whole document ---	1-17
P,X	WO 96 36239 A (C MCLACHLAN) 21 November 1996 see the whole document ---	1-17
P,X	THE LANCET , vol. 348, no. 9032, 5 October 1996, LONDON GB, pages 926-928, XP002030854 M G CAVALLO ET AL.: "Cell-mediated immune response to beta casein in recent-onset insulin-dependent diabetes; implication for disease pathogenesis" see the whole document -----	1-17

International application No.

PCT/EP 96/05846

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: 15-17
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 15 to 17 are directed to a method of treatment of the human body, the search has been carried out and based on the alleged effects of the products.
2. Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte	nal Application No
PCT/EP 96/05846	

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9517518 A	29-06-95	FR 2714390 A AU 1319795 A CA 2179738 A EP 0736102 A	30-06-95 10-07-95 29-06-95 09-10-96
WO 9406306 A	31-03-94	AU 677230 B AU 4987393 A EP 0661927 A JP 8501688 T NZ 255608 A	17-04-97 12-04-94 12-07-95 27-02-96 28-10-96
WO 9102539 A	07-03-91	FR 2650955 A AT 112683 T DE 69013350 D DE 69013350 T EP 0487619 A ES 2064759 T	22-02-91 15-10-94 17-11-94 23-03-95 03-06-92 01-02-95
WO 9614577 A	17-05-96	AU 3939595 A NZ 295774 A	31-05-96 24-03-97
WO 9636239 A	21-11-96	AU 5510896 A	29-11-96

09 / 095639

- 1 -

PRODUCT DERIVED FROM MILK SUBSTANTIALLY FREE OF BETA CASEIN FROM NON-HUMAN MAMMALS AND RELATIVE USE

Ins C¹

Field of invention

The present invention is related to a product derived from milk substantially free of beta casein from non-human mammals. The invention is also related to the use of such a product especially in terms of its application in relation to diet, particularly for early infancy, in the prevention of insulin-dependent diabetes.

Prior art

10 The technique of obtaining products, especially food products, for early infancy is well-known, starting from non-human milk, such as cow's, sheep and goat's milk. The basic component of milk is characterized by casein, which in basic terminology represents a group of proteins obtainable by milk precipitation at acid pH and up to room 15 temperature, specifically pH 4.6 and 20°C. Caseins represent approximately 80% of total cow's milk proteins and 40% p/v human milk. Casein can be sub-divided into three main groups: alpha, beta and kappa. There is also a fourth group, represented by gamma casein, which is derived from beta casein following the removal of the first 20 twenty-two amino acids. Therefore, for the present invention, gamma casein will be considered as part of beta casein.

Beta casein represents approximately 70% p/v of all casein present in human milk, whereas in cow's milk, it represents approximately 25% p/v. Of bovine beta casein, several genetic variants are known and have 25 been characterized, including A1, A2, A3, B, C, D and E. For the industrial production of milk, mainly the genetic variant of milk A1 has been favored to increase the amount of milk produced. This

- 2 -

variant, which contains more proteins than others, has been obtained from various selected animals, in particular cows. By gene data sequencing analysis, the amino acid sequence in position 63-68 has been identified for cow's beta casein A1, corresponding to the 54-59 sequence of human beta casein. A similar situation has been discovered with regard to the variant A2. Both variants A1 and A2 of beta casein also show sequence homology in that region (at least 90 percent) with a specific protein of insulin-producing cells in the pancreas (GLUT2). According to the inventor, the sequence 63-68 of A1 and A2 beta casein and, more generally, the analogue sequences of other types of casein, such as A1, A2, A3, B, C, D and E, elicit an immune response via production of anti beta casein antibodies and lymphocytes which recognize such sequences. For newborns and infants in the first months of life, a diet containing these immunogenic caseins might induce a specific immune response to GLUT2 in the insulin-producing cells of the pancreas by a mechanism of molecular mimicry with the homologous sequence of beta casein. On the basis of such a hypothesis, a study has been carried out, aiming to obtain bovine milk products substantially free of non-human beta casein and, more specifically, beta casein containing products from non-human mammals that do not result to be immunogenic with respect to the GLUT2 protein because of absence of such sequence homology.

Summary of the invention

The present invention is related to a product derived from milk or milk itself, substantially free of non-human beta casein with immunogenic characteristic as specified in prior art.

Another object of the invention is a milk-derived product or milk

- 3 -

itself comprising at least one beta casein modified from non-human mammals without the immunogenic characteristic mentioned above.

Another object of the invention is the use of such a product, in relation to diet.

5 Another object of the invention is the use of a product from milk or milk itself, substantially free of non-human mammals beta casein in order to obtain a food for the early infant diet for the prevention of insulin-dependent diabetes.

Further objects of the invention will be evident from the detailed

10 description of the invention

Detailed description of the invention

In the attached description the amino acid sequences of importance according to the invention will be underlined. The word "substantially free" will indicate the presence of the substance (s) to which it

15 refers in amounts ranging between 0 to 10% b.w.

The amino acid sequence of interest for the present invention is

described hereafter. As mentioned above, according to the inventor there is a correlation between exposure to cow's milk and the development of insulin-dependent diabetes due to molecular mimicry

20 between the amino acid sequences of beta casein A1 and A2 and a specific sequence of the GLUT2 protein found in the insulin-producing cells. Such a sequence has been identified as follows:

Pro-Gly-Pro-Ile-His-Asn (where the underlined sequence is SEQ ID NO:1)

for the A1 beta casein inserted in the larger fragment: Ser-Leu-Val-

25 Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn (SEQ ID NO:3).

As already stated, such a sequence is also present in gamma casein.

Other sequences corresponding to immunogenic peptides of beta casein

- 4 -

which are different from those mentioned above are given as examples.

Cow's beta casein A2 from bos taurus (63-68), Pro-Gly-Pro-Ile-Pro-Asn (where the underlined sequence is SEQ ID NO:2) inserted in the larger fragment: Ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn (SEQ ID NO:4)

Beta casein from bos indicus (63-68): Pro-Gly-Pro-Ile-Pro-Asn (underlined sequence SEQ ID NO:2).

In comparison, human beta casein has the following sequence (48-59):

Ser-Leu-Val-Tyr-Pro-Phe-Val-Glu-Pro-Ile-Pro-Tyr (SEQ ID NO:6). The peptide fraction relevant to the present invention has been identified as (54-59): Val-Glu-Pro-Ile-Pro-Tyr (where the underlined sequence is SEQ ID NO:5). The peptide sequences of GLUT2, the glucose transporter inside insulin-producing beta cells in the pancreas, are the following:

- 15 (409-420) Ser-Phe-Phe-Glu-Ile-Gly-Pro-Gly-Pro-Ile-Pro-Trp
(412-423) Glu-Ile-Gly-Pro-Gly-Pro-Ile-Pro-Trp-Phe-Met-Val
(414-425) Gly-Pro-Gly-Pro-Ile-Pro-Trp-Phe-Met-Val-Ala-Glu

The inventor suggests that the sequence of A1, B and C beta casein and gamma casein, Pro-Gly-Pro-Ile-His (SEQ ID NO:1), and the larger fragments containing it, such as the sequences of beta casein A2, A3 and E, Pro-Gly-Pro-Ile-Pro (SEQ ID NO:2), are responsible for the induction of an immune response towards beta casein which, by cross reactivity, would be directed towards the homologous sequence of GLUT2, causing damage to the cells that produce insulin.

- 25 Therefore to produce a milk or in general, food product comprising non-immunogenic beta casein for administration in diets, particularly to newborns and in early infancy, would be a preventive approach

- 5 -

against insulin dependent diabetes.

All caseins which do not contain the sequence Pro-Gly-Pro-Ile-His (SEQ ID NO:1) or Pro-Gly-Pro-Ile-Pro (SEQ ID NO:2) are not considered pathogenic and, therefore, can be used to produce a dietary product in accordance with the present invention:

- some or all amino acids present in the above sequence are modified;

- beta casein does not contain such a sequence (e.g., it has been removed)

10 - beta casein is modified in that such a sequence is substituted with a sequence of human beta casein;

All modifications can be made by applying the well-known technique of genetic engineering and the classic biological technique of cross-selection, as described in patent WO 93/04171.

15 The milk obtained, comprising casein modified as stated above, can be administered as such or can be treated with known methods, as the casein(s) involved can be separated and used to produce food and pharmaceutical products.

In particular, the products including such casein can be used for 20 administration in early infancy and later on as a diet for the prevention of insulin-dependent diabetes.

It is preferred that, in products according to the present invention, concentrations of A1 and/or A2 and/or other immunogenic beta caseins, in particular those with the sequence Pro-Gly-Pro-Ile-His (SEQ ID NO:1) or Pro-Gly-Ile-Pro (SEQ ID NO:2), do not represent more than 10% b.w. of the final product.

The food products of the invention can be, for instance, pasta, milk

- 6 -

and milk-derived products and proteins, such as those added to food, all of which are already in the marketplace, the modification being the substitution of the immunogenic caseins with the caseins of the present invention.

5 Also part of the present invention are vegetable and/or synthetic proteins, such as those derived from soya. According to the teaching of the invention, it is possible to produce a pharmaceutical or food product, especially for early infancy, substantially free of beta casein, with the amino acid sequence Pro-Gly-Pro-Ile-His (SEQ ID NO:1)

10 or Pro-Gly-Pro-Ile-Pro (SEQ ID NO:2), or where such sequences are less than 10% of the final weight of the product. It is also possible to produce a food product or a milk according to the following alternatives:

- where the beta casein is lower than 10% b.w. or the beta casein
15 comprising the amino acid sequence Gly-Pro-Ile-His (SEQ ID NO:7) or Gly-Pro-Ile-Pro (SEQ ID NO:8) is lower than 10% by w.

- substantially free of beta casein comprising the amino acid sequence Gly-Pro-Ile-His (SEQ ID NO:7) or Gly-Pro-Ile-Pro (SEQ ID NO:8) and integrated with peptides derived from the hydrolysis of animal,
20 vegetable and/or synthetic proteins, and lacking these above sequences and mixtures thereof (FR 86-00325, WO 94/06306, WO p (02539));.

- where the beta casein comprising the amino acid sequence Gly-Pro-Ile-His (SEQ ID NO:7) or Gly-Pro-Ile-Pro (SEQ ID NO:8) is lower than 10% b.w. and integrated with peptides coming from hydrolisis of animal
25 and/or vegetal and/or synthetic proteins lacking such sequences and mixtures thereof;

- where the beta casein is lacking the amino acid sequence Gly-Pro-

- 7 -

Ile-His (SEQ ID NO:7) or Gly-Pro-Ile-Pro (SEQ ID NO:8) in that it has been obtained from animal species genetically not producing proteins with such sequences;

- milk naturally lacking beta casein, produced by genetically modified animals according to patent WO 93/04171;
- milk comprising human beta casein obtained from genetically manipulated microorganisms or animals, such as those described in the above mentioned patent.

The protein fractions can be derived from chemical-physical treatments of milk and from lyophilized casein, for instance by differential solubility, liquid-liquid extraction, membrane separation, chromatographic separation, as described in patents FR 86-00325 and WO92/00017.

The integrations can be carried out by using recombinant beta casein produced with one of the well-known cloning methods, using yeast, bacteria, fungi or transgenic animals, such as those described in patent WO 93/04171.

A process for removing beta casein from milk is the chromatographic process, as described below.

By means of such a process the beta casein is separated, starting from acid casein, and by means of chromatography in two steps, the remaining fractions of alpha and kappa casein will be obtained.

The process can be optimized using the knowledge already available in the field. Such a process includes the use, as basic phase, of a resin of ionic exchange, for example Sepharose[®] from Pharmacia, with the concentration also in columns. The mobile phase is constituted by Buffer A:

- 8 -

- Sodium acetate with concentration not less than 10mM;
- urea at concentration not less than 2M;
- pH between 5 and 6.

The acid casein can be dissolved in Buffer A at pH not less than 6,
5 with the addition of a specific reducing agent, DTT, (Ditiotriitol).
The mixture should be left under for 24 hours, brought to pH between 5
and 6 and placed in columns. The beta casein fraction does not
interact with the resin and is eluted in 0M NaCl. It is not necessary,
therefore, to carry out stages of increasing ionic concentration.
10 considering that the process at hand merely involves a simple
separation of beta casein from the other fractions, which will be
collected in isocratic by eluting with buffer B:

- Sodium acetate at concentration no less than 10mM;
- urea at concentration no less than 2M;
- 15 - 0.8 M NaCl;
- pH between 5 and 6.

The fractions are later dialfiltrated to eliminate urea and other
salts; after concentration, caseins are collected by acid
precipitation and the obtained wet product is lyophilized.
20 Brief description of the drawings.
Fig. 1 is the chromatogram relating to the initial load of Example 1;
Fig. 2 illustrates a chromatographic peak relating to the beta
casein;
Fig. 3 refers to the absence of the beta casein in the chromatogram.
25 The following examples are to be considered as illustrative of such a
technique, therefore they should be not considered a limitation of the
gist of the present invention.

- 9 -

Example 1:

Separation of beta casein from acid casein and collection of the remaining alpha and kappa casein fractions.

Preparation

- 5 - 200 g acid casein + 3000 ml Buffer C: 20 mM sodium acetate, 4M urea, 10 mM (ditioltreitol) DTT pH 7.

Casein should be slowly dissolved in the buffer, keeping pH 7 with 2M NaOH at each addition.

Leave under stirring at 5° C for approximately 12 hours.

- 10 Filtrate the solution in pre-filter Millex AP-50 (Millipore)

Wash the prefilter with 1000 ml of Buffer C and collect.

Bring the load (4000 ml) to pH 5.5 with HCl 6M and adjust the ionic strength (2.2 mS) to 4.5 mS.

Preparational Chromatography

- 15 - FPLC Waters 600 Controller

- Revelator: Perkin Elmer UV/VIS Spectrophotometer Lambda 3B 280 nm

- Column: XK 50 Pharmacia (maximum pressure 3 bar), Ø 5 cm, height 100 cm

- Resin: S-Sepharose Pharmacia height 85 cm, volume 1670 ml

- 20 - Eluents: Buffer A Sodium acetate 20 mM

Urea 4M

pH 5.5, ionic strength 1.5 mS

Buffer B Sodium acetate 20 mM

Urea 4M

- 25 pH 5.5

Sodium chloride 1 M ionic strength 57.1 mS

All buffers are filtrated by using a Millex pre-filter AP 50 bound in

- 10 -

series with filter 0.45 µm Millipak 20 (Millipore).

- Temperature: Room temperature

- Conditioning: ≈ 8000 ml

Buffer A: 97% Buffer B: 3% ionic strength mix:

5 4.5 mS

Flow: 30 ml/min P=42 PSI

Time: 4 hours, 25 minutes

- Loading: 200 g acid casein dissolved in Buffer C (total volume 4000 ml)

10 Flow: 20 ml/min P=50 PSI

Time: 3 hours, 20 minutes

- Elution: First Stage (isocratic) ≈ 8000 ml
 Buffer A:97% Buffer B:3%

15 Flow: 30 ml/min P=42 PSI

Time: 4 hours, 25 minutes

Second Stage (isocratic) 9000 ml

Buffer A:20% Buffer B:80%

Flow: 30 ml/min P=42 PSI

20 Time: 5 hours

Example 2 - Control test

An amount of the product from example 1 is tested by chromatography to evaluate the absence of beta casein in the isocratic of the second stage. Such absence is confirmed as demonstrated by the chromatogram 25 of figure 3. By comparison in figure 1, the chromatogram relating to the initial load is illustrated, whereas in figure 2 the peak relating to the presence of beta casein only derived from the elution of the first stage is represented.

- 11 -

Analytic chromatography

- 5 - HPLC Perkin Elmer Biocompatible Binary Pump 250

- Revelator: Perkin Elmer LC95 280 nm

- Column: Mono S HR 5/5 Pharmacia

- Loop: 100 µl

- Eluents: Buffer A* Sodium acetate 20 mM
 Urea 6 M
 pH 5

 Buffer B* Sodium acetate 20 mM
 Urea 6 M
 Sodium chloride 1 M
 pH 5

- 10 - Temperature: Room temperature
- Flow: 1 ml/min
- Conditioning: 8'00" Buffer A* 100% Buffer B* 0%
- Elution: gradient 5'00" Buffer B* 50% Buffer B* 50%
 (increase of B* 1% min)
 isocratic 2'00" Buffer A* 50% Buffer B* 50%
15 isocratic 5'00" Buffer A* 0% Buffer * 100%

- 12 -

Example 3

The product of Example 1 has been purified from urea by the following method of diafiltration.

- Ultrafiltration S.G.I.
 - 5 - Cellulose membrane S-10 10.000 Da Amicon
 - Buffer of dialysis: demineralized water
sodium acetate 10 mM pH?7
ionic strength 0.8 mS
total volume 250 l (5 washes)
 - Permeate flow: 32+37 l/h
 - Temperature: 10°C
 - Product concentration 50 l up to 20 l
- 10 The product has been tested to verify the absence of urea as follows:

Urea Test

- UV method (Boehringer Mannheim)
- Spectrophotometer: Lambda 3B 340 nm (Perkin Elmer)

Reagents	Blank	Sample
Solution 1	1.00 ml	1.00 ml
Sample solution	---	0.10 ml
Solution 2	0.02 ml	0.02 ml
Bidistilled water	2.00 ml	1.90 ml

Incubate 5' at 20-25°C; read the absorbance (A1)

Solution 3 0.02 ml 0.02 ml

Incubate 20' at 20-25°C; read the absorbance (A2)

Solution 1 = Triethanolamin buffer, pH 8.2 oxoglutarate, NADH
Solution 2 = Urase
Solution 3 = Dehydrogenase glutamate

- 13 -

The lyophilization is carried out on the product free of urea, by using a Christ model Beta 1-16 equipment.

- 14 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: BIOSISTEMA di Pier Luigi Sarapani & C. Sas
- (B) STREET: Via Per Luco dei Marsi
- (C) CITY: Avezzano
- (D) STATE: L'Aquila
- (E) COUNTRY: Italy
- (F) POSTAL CODE (ZIP): 67051

(ii) TITLE OF INVENTION: Product derived from milk substantially free of beta casein from non-human mammals and relative use.

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: IT RM 95 A 000850
- (B) FILING DATE: 27-DEC-1995

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Pro Gly Pro Ile His
1 5

- 15 -

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Pro Gly Pro Ile Pro
1 5

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ser Leu Val Tyr Pro Phe Pro Gly Pro Ile His Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ser Leu Val Tyr Pro Phe Pro Gly Pro Ile Pro Asn
1 5 10

- 16 -

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Val Glu Pro Ile Pro
1 5

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Ser Leu Val Tyr Pro Phe Val Glu Pro Ile Pro Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Gly Pro Ile His
1

- 17 -

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Gly Pro Ile Pro
1